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PURIFICATION AND ANALYSIS OF A RECOMBINANT HUMAN ANTI-CHOLERA TOXIN B ANTIBODY

Jessica Dang
Suzanne Kracke
GEO-CENTERS, INC.
Rockville, MD 20852

Peter A. Emanuel
Michael J. Gostomski
Darrel E. Menking
James J. Valdes
RESEARCH AND TECHNOLOGY DIRECTORATE

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GEO-CENTERS, INC.

Aberdeen Proving Ground, MD 21010-5423

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Dang, Jessica; Kracke, Suzanne (GEO-CENTERS, INC.); Emanuel, Peter A.; Gostomski, Michael J.; Menking, Darrel E.; and Valdes, James J. (ERDEC)

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A large semi-synthetic recombinant antibody library was constructed based on a human anti-tetanus library. The library was used to isolate a human anti-cholera toxin B antibody clone, which is expressed in XL-1 Blue *E. Coli* bacteria. The clone, B-27 cholera Fab, was expressed in small scale and purified by a two column procedure, which uses the histidine affinity tag, which was engineered into the protein. The purified cholera Fab was tested by ELISA to confirm specificity and by surface plasmon resonance to determine affinity binding constants.

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PREFACE

The work described in this report was authorized under Project No. 10262384A553, Non-Medical CB Defense. This work was started in November 1996 and completed in July 1997.

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PURIFICATION AND ANALYSIS OF RECOMBINANT HUMAN ANTI-CHOLERA TOXIN B ANTIBODY

1. INTRODUCTION

In 1993, over 360,000 cases of cholera infection were reported to the World Health Organization.[a] Infection results in severe fluid loss and is life threatening if untreated. Cholera toxin B is a pentamer that binds to and enters cells, such as intestinal epithelial cells, which have the charged glycolipid, ganglioside G_{M1} on their surface.[1,2] Cholera toxin catalyzes the transfer of ADP ribose from intracellular NAD^+ to the alpha subunit (α_s) of a trimeric G protein. ADP ribosylation inhibits the self-inactivating mechanism of α_s, rendering it unable to hydrolyze its bound GTP. Constitutively active α_s causes prolonged elevation of cAMP, resulting in a large efflux of Na^+ and water into the gut and subsequent severe diarrhea.[1,2]

In this report, we describe the purification and kinetic analysis of a recombinant human anti-cholera toxin B antigen binding fragment (Fab), B-27. The B-27 antibody fragment was isolated from six pooled anti-tetanus-synthetic human antibody libraries which were randomized within the complementarity determining region 3 (CDR3) of the heavy and light chain genes. This randomization was performed in an effort to increase the binding diversity of the combinatorial library and result in the isolation of higher affinity binding to the target antigens. B-27 was subcloned into an affinity purification vector and purified for characterization. The resulting protein has an extremely high binding affinity, but induction of the protein appears to be toxic to the bacterial expression system.

2. MATERIALS AND METHODS

2.1 Preparation of Biomaterials

B-27 anti-cholera Fab, cloned into the T7 expression vector pWPY501 in the *E. coli* cell line B834 (glycerol stock), and purified B-27 cholera Fab were provided by Scripps Research Institute, La Jolla, CA. Media, LB^{carb} (Luria-Bertani, Difco Laboratories, Detroit, MI; 100μg/ml carbenicillin), SOC medium (GIBCO/BRL), and TB^{carb} (Terrific Broth, GIBCO/BRL 50μg/ml carbenicillin, 20mM $MgCl_2$) were prepared and autoclaved. Restriction enzymes, *Eco*RI and *Spe*I, were obtained from Boehringer Mannheim (Indianapolis, IN); T4 DNA ligase was purchased from USB/Amersham (Arlington Heights, IL). All other chemicals were of the highest quality and purchased from Sigma Chemical Co. or Boehringer Mannheim.

[a] Duty Officer, Personal Communication. Centers for Disease Control, Atlanta, GA. June 1997.

2.2 Subcloning

An LB^{carb} plate was streaked with B-27 pWPY501 in B834 cells and incubated overnight at 37°C. A single colony was inoculated in LB^{carb} and plasmid DNA was extracted the next day. One hundred micrograms of B-27 pWPY plasmid DNA was cut with 200 units of *Eco*RI and *Spe*I in order to isolate the B-27 Fab gene insert from the original cloning vector, pWPY501. The pHist expression vector containing a 6X histidine tag was prepared in the same manner. The insert and vector DNA were excised from a 1% TAE agarose gel, eluted, and precipitated with 1/10 volume of 3M sodium acetate, 2 volumes of 100% ethanol, 20μg glycogen at -20°C for 3 hours. Five hundred nanograms of *Eco*RI/*Spe*I-cut B-27 were ligated into 450ng of *Eco*RI/*Spe*I-cut pHist with 10 units of T4 DNA ligase at 16°C for 20 hours. Ligase was inactivated by incubating reaction at 70°C for 10 minutes. Ligated DNA was precipitated as described, centrifuged, dried, and resuspended in H_20. DNA was then electroporated at 1.8kV (BioRad E. coli purser) into 40μl of XL1-Blue electrocompetent cells. The 0.1 cm gap electroporation cuvette was washed with 3ml of SOC medium, and incubated at 37°C for one hour. Transformation reaction was diluted with 10ml of TB^{carb}, plated on LB^{carb} plates in four serial dilutions (10μl, 1μl, 0.5μl, O.1μl), and incubated overnight at 37°C. The next day, eight colonies were inoculated in 10ml LB^{carb}. Glycerol stocks were made of all colonies selected (750μl of overnight bacterial culture plus 750μl 70% glycerol). Plasmid DNA was extracted (Promega Wizard Mini Prep Kit, Promega Corporation, Madison, WI), and colonies screened for the correct insert and vector via restriction enzyme digest.

2.3 Anti-cholera Toxin Direct ELISA

Cell protein extract was prepared by streaking an LB^{carb} plate with a glycerol stock of the B-27 construct. A single colony was inoculated in LB^{carb} and incubated for 6 hours at 37°C. Fab expression was induced by addition of 1mM or 5mM isopropyl ß-D-thiogalactopyranoside (IPTG) and incubated overnight at 30°C. Culture was centrifuged (3500g, 10 min), supernatant decanted, and the resulting cell was resuspended in 1ml TBS, sonicated, and centrifuged as described. Whole cholera toxin or subunit B was used as antigens to characterize the specificity of recombinant cholera Fab.

Direct ELISA assays were performed using 96 well microtiter plates coated overnight at 4°C with 50μl cholera toxin (2μg/ml in 0.1M bicarbonate buffer). After incubation, the plates were washed three times with TBS (Tris-buffered saline, 20mM Tris-HCl, pH 7.6, 50mM NaCl), followed by blocking with 10% skim milk in TBS for two hours at room temperature. After washing, three different volumes of B-27 cell protein extract were added to alternating wells of the ELISA plates and incubated for two hours at 25°C. Purified B-27 cholera Fab, diluted 1:1000, 1:2000, and 1:3000 in TBS was used as a positive control. The plates were then washed as before with ELISA wash buffer. Alkaline phosphatase-conjugated goat anti-human antibody (Pierce Chemical Company, Rockford, IL) diluted 1:1000 in 10% skim milk/TBS was added to each well of the ELISA plate and incubated for two hours at 25°C. The plates were washed three

times with TBS. A pNPP tablet (20mg p-nitrophenyl phosphate) was added to 20 ml developing solution (5OmM sodium carbonate pH 9.6, 5mM $MgCl_2$) and 100μl was added to each well. Optical density at 405nm (OD_{405}) of each well was determined using an ELISA plate reader (Dynatech, Chantilly, VA). The positive cutoff for this assay was calculated from the adjusted OD_{405} of the mean plus three standard deviations of a negative control consisting of culture media without Fab.

2.4 Antibody Scale-up

A single colony of B-27 pHist was selected from an LB^{carb} plate and inoculated in 150ml TB^{carb} at 37°C overnight. Ten ml of overnight bacterial culture was added per liter of growth media and incubated at 37°C for several hours until an OD_{600} 0.5-0.7 was obtained. Fab expression was induced by the addition of 1mM IPTG. Culture was then incubated at 30°C for 18 hours. Induced, overnight culture was centrifuged (5,000g, 15 min), supernatant was decanted, and cell pellet paste was resuspended in 80ml/L of culture in nickel column sonication buffer (50mM K_3P0_4, pH 8, 300mM NaCl, 200μM PMSF). Cells were disrupted by ultrasound (high intensity Ultrasonic Processor, Model GEX600) and then centrifuged at 30,000g for 30 min. Cell pellet extract was decanted and filtered through 0.45μm and 0.22μm membrane filters.

2.5 Fab Purification

Twelve ml of nickel nitrilotriacetic acid sepharose (Ni-NTA, Qiagen Inc., Chatsworth, CA) was prepared by exchanging the storage buffer with nickel column sonication buffer. Cell pellet extract was incubated with resin at 4°C overnight. Extract/resin mixture was centrifuged (5000g, 15 min), supernatant was decanted. The resin was washed in batch 3 times with sonication buffer, once with high salt wash buffer (1.5M NaCl), 3 times with nickel column wash buffer (5OmM K_3P0_4, pH 6, 10% glycerol, 200mM NaCl), and three times with wash buffer supplemented with 5mM imidazole. Samples of each wash were collected in order to monitor when non-specifically bound proteins were removed from the nickel resin. Bound Fab was eluted with elution buffer (5OmM K_3P0_4 pH 6, 10% glycerol, 300mM NaCl, 5OOmM imidazole) and dialyzed overnight in 10mM K_3P0_4, pH 6.8, 200μM PMSF at 4°C. A 20ml Macro-Prep ceramic hydroxyapatite (CHT, Bio-Rad Type 1, 40μM particle size) column was prepared according to manufacturer's specifications. Column was equilibrated with three columns of loading buffer, 10mM K_3P0_4 pH 6.8 (Buffer A). Overnight diasylate was filtered with a 0.22μM membrane filter, loaded onto the CHT column, and washed with 4 column volumes of Buffer A. Fab was eluted with a linear gradient of Buffer B (1M NaCl) and fractions collected (Bio-Rad Automated Biologic Workstation). CHT column was regenerated with 400mM K_3P0_4, washed with ddH_20, and column stored in 20% ETOH. All buffers for CHT chromatography were filtered, degassed, and chilled to 4°C.

2.6 Analysis of Fab Purity

Following CHT column purification, Fab purity was analyzed by SDS polyacrylamide gel electrophoresis under non-reducing conditions followed by staining with Coomassie blue R-250 and western blot analysis. Western blot was probed with a 1:1000 dilution of goat anti-human IgG-alkaline phosphatase-conjugated antibody in TBS/10% skim milk and incubated at room temperature for 2 hours. Blot was washed 3 times with TBS and bands visualized with developing solution (100mM Tris pH 9.25, 5mM $MgCl_2$, 100mM NaCl, 330μg/ml nitro-blue tetrazolium and 165μg/ml 5-bromo-4-chloro-3-indolyl-phosphate).

2.7 BIAcore Kinetic Analysis

Fab was quantitated by standard Bradford assay (Bio-Rad) and dialyzed against HBS (0.1M Hepes pH 7.4, 0.15M NaCl, 3mM EDTA). Lyophilized cholera toxin, subunit B was resuspended to 20μg/ml in 5mM sodium maleate buffer (pH 6). All solutions were filtered through a 0.22μM filter. Ninety seven resonance units (RU) of cholera toxin were covalently immobilized to a CM5 sensor chip by amine coupling using N-hydroxysuccinimide esters. Cholera Fab was passed over the chip at 17μl/min at the indicated concentrations. Kinetic determination was performed using BIA Evaluation 2.1 and values were averaged constants from three separate immobilizations.

3. RESULTS

Six semi-synthetic anti-tetanus toxin human Fab libraries constructed by Dr. Carlos Barbas (Scripps Institute, La Jolla, CA) were combined. Dr. Larry Lo and Dr. Kim Janda (Scripps Institute, La Jolla, CA) randomized various amino acids in the CDR3 regions of the heavy and light chains in an attempt to increase the binding diversity of the clones. Cholera toxin B was immobilized in microtiter wells and used for the selection of bacteriophage displaying Fab antibody as a fusion with the cpIII protein on the viral surface. Biopanning was performed essentially as described by Barbas III *et al.*[5]

B-27 anti-cholera Fab was initially received cloned into the T7 expression vector pWP501 and contained within the *E. coli* cell line B834. The recommended procedures advised the isolation of Fab from extracts using an anti-human Fab affinity column eluted with citric acid. The anti-human Fab column was constructed by immobilizing 2mg of goat anti-human Fab polyclonal antibodies on 2ml of protein G sepharose. The protein G/polyclonal beads were then subjected to covalent modification using dimethylpimelimidate according to methods outlined by Harlow *et al.*[6] The column was used to successfully isolate the cholera antibody from 1L shaker cultures following induction with IPTG.

Although the protein was relatively pure following immunoaffinity purification, the yield was not as expected and smaller degradation products co-purified with the Fab. Because the proteins were 30kD in size and were detected in an anti-human Fab western blot, it was assumed

that these were either heavy or light chains that were not associated into functional antibody (data not shown). The construction of larger immunoaffinity purification columns is expensive and their stability is limited, therefore, it is desirable to purify the antibody through the use of standard chromatography resins.

The Fab gene insert was then transferred into a vector which added a six histidine affinity tag to the carboxyl terminus of the heavy chain. This affinity tag has been shown not to interfere with the binding of the antibody in various other constructs and our analysis showed that this assumption held true for B-27. Initial purifications with Ni-NTA resin revealed that the Fab was purified by passage over the affinity resin, but only by a factor of 2000 fold.

Purification was achieved by passage over the CHT column. This resin has the advantage of high specificity and resolution while being capable of withstanding high flow rates. Initial attempts to elute the protein with an increasing gradient of phosphate ions did not result in considerable purity of the final product.

In order to optimize the elution from CHT, the Fab was subjected to isoelectric focusing using the PHAST gel system (Pharmacia). By electrophoresing the immunoaffinity purified Fab through IEF gels and comparing to IEF standards, we were able to ascertain that the isoelectric point (pI) of the Fab was 8.0.

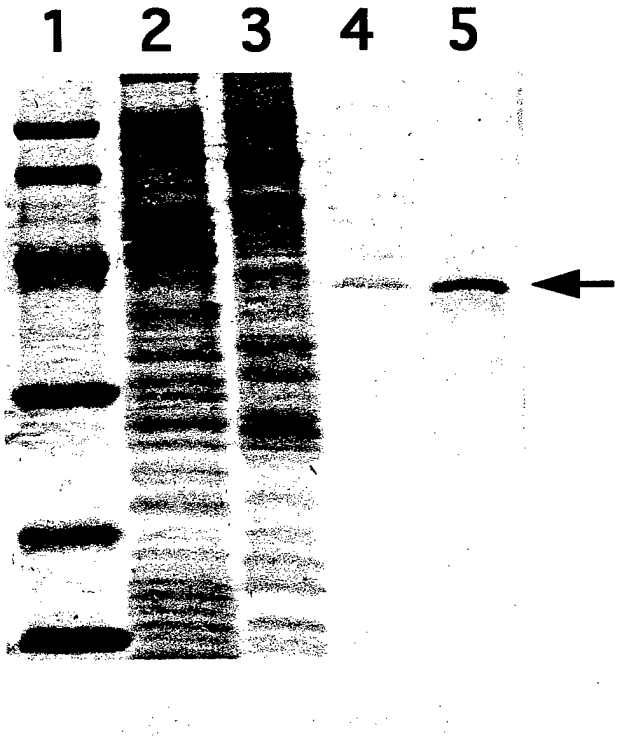

Figure 1: 12% SDS-PAGE analysis of purified cholera Fab protein stained with Coomassie Blue. Lane 1: Bio-Rad Low molecular weight markers; Lane 2: 4µl Crude cell lysate; Lane 3: 10µl Ni-NTA column eluate; Lane 4: 20µl CHT column purified anti-cholera Fab; Lane 5: 20µl Western blot of CHT column pure anti-cholera Fab using anti-human Fab.

Because the protein had a pI above 7.0, an elution gradient of increasing amounts of NaCl was chosen. This alternative is an attractive means of purifying basic proteins from the CHT resin. Flask cultures of B-27 were grown and extract was

purified through a Ni-NTA column (Figure 1, lane 3). The resulting elution with imidazole was pooled and passed over a CHT column. The Fab consistently eluted off the CHT column at approximately 650mM NaCl with an estimated purity of 95% (Figure 1, lane 4). Western blot analysis confirmed that the purified protein was recognized as human Fab which had been expressed in bacterial cells (Figure 1, lane 5). The binding specificity was confirmed by ELISA against cholera toxin B as described in Materials and Methods (data not shown). The resulting production and purification bioprocess is summarized in Figure 2.

Cholera toxin B was immobilized to a CM5 sensor chip for affinity constant determination. The BIAcore™ biosensor uses surface plasmon resonance to detect changes in mass at the binding surface. As B-27 binds to the immobilized toxin, the biosensor will detect changes in mass which are measured as increasing resonance units (RUs) in real time (seconds). Figure 2 shows 8 different concentrations of B-27 ranging from 25 to 200nM which were passed over a sensor chip to which cholera toxin B had been immobilized. Analysis of the binding curves using BIAevaluation 2.1 software calculates the k_{on} rate of $3x10^4$ M/sec and a k_{off} rate of $4x10^{-4}$ M^{-1}. The ratio of k_{off} and k_{on} gives an equilibrium dissociation constant (K_{eq}) of 60nM.

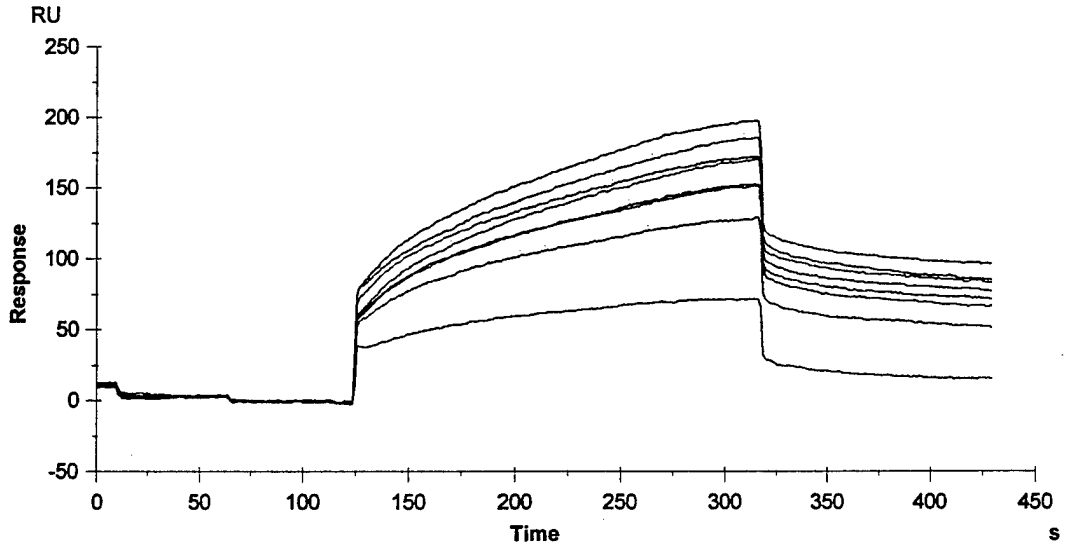

Figure 2: BIAcore Kinetic Analysis of anti-cholera Fab. Cholera toxin, subunit B in 5mM maleate buffer (pH=6) was immobilized to a CM5 sensor chip using amine coupling. Purified B-27 pHist cholera Fab at 25nM, 50nM, 75nM, 85nM, 100nM, 135nM, 150nM, 175nM, and 200nM was passed over the surface at 17µl/min for 450 seconds. The binding reaction is measured in resonance units (RUs) along the y-axis and the time in seconds along the x-axis.

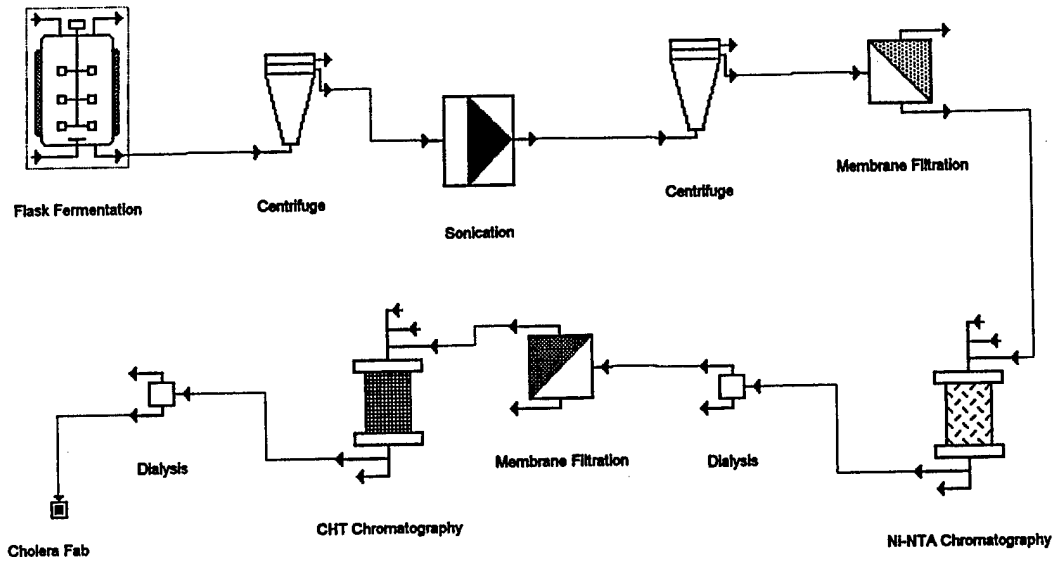

Figure 3: Bio-process for the production of cholera Fab. Cholera Fab was expressed in bacterial cultures and the cells pelleted following induction by IPTG. Cells were disrupted in 50mM K_3PO_4 pH 8.0 by sonication and the debris were pelleted and filtered through a 0.22μm filter. The extract was purified by affinity chromatography using Ni-NTA resin and eluted with 0.5M Imidazole, 0.3M NaCl, 50mM K_3PO_4. The eluted fraction was dialyzed into 10mM K_3PO_4 pH 6.8 and passed over a 20ml CHT column. The column was washed and the protein eluted with a 0-1M NaCl gradient in 10mM K_3PO_4 pH 6.8. The pooled fractions were dialyzed into 10mM NaMaleate pH 6.0 for BIAcore analysis.

4. DISCUSSION

The need for a more economical means of mass producing antibodies for detection of biological warfare agents was addressed by the selection of recombinant antibody fragments (Fabs) from naive semi-synthetic human combinatorial libraries. The development of this technology is advantageous for rapid, large-scale production because it permits the use of standard biomanufacturing techniques using bacterial fermentation.[7] The most recent technology is focusing on the construction of larger libraries which may approach the natural diversity of the human immune system.[4,8,9]

The ability to alter the selection process gives an additional level of control which allows the selection process to be driven towards a particular antigen or epitope.[10] Antibody producing clones are available in prokaryotic systems as a double stranded phagemid. The genetic manipulation of this plasmid DNA is straightforward and provides the capability to alter the affinities by mutagenesis or by chain shuffling.

Downstream processing has been simplified by the insertion of an affinity tag at the nucleotide level which allows the Fab to be expressed as a fusion protein with a histidine hexapeptide tag on the carboxyl terminus of the heavy chain. Future clones will also contain a

FLAG or c-myc tag for easy immunochemical identification and tracking of clones. One proposed project currently under review is the production of Fabs as fusions with marker proteins in order to track antibody production visually during purification.[11] Antibody constructs are also being transferred to other expression systems such as Chinese hamster ovary (CHO) cells and for secretion in the milk of transgenic animals in an effort to achieve higher yields.

Requirements for antibodies with particular specificities and affinities have not been met using standard technologies.[12] Biosensor applications and catalytic immuno-enzyme assays require antibodies with specific epitope recognition and affinities which can be best produced by a recombinant antibody approach. Bacterial expression of Fab fragments gives lower yields than hybridoma cultures, but is far less expensive to scale-up.[10,13] Large-scale growth and purification of cell lines is faster, giving a finished product in as little as one week. The power of this technique lies in the vast numbers of clones that can be screened and enriched in order to isolate unique specificities, and in the ease with which these clones can be genetically manipulated. As this technique gains wider acceptance for use in isolating immunoglobulins, significant improvements will give the researcher greater flexibility in immunodiagnostics and therapeutics.

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